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Characterization of a native ethanol producing *Zymomonas* sp. isolated from natural environments of Mazandaran province, North Iran

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ABSTRACT

Ethanol is a renewable and safe fuel and it is mainly produced based on microbial fermentation. The present study aims to isolate and identify ethanol producing *Zymomonas* spp. from natural environments with characterization, optimization and evaluation of their ethanol productivity. Samples from various fruits and sap of plants were screened for ethanol producing bacteria on RM medium. Ethanol producing isolates were selected for characterization. In addition, bacterial growth and ethanol production conditions were optimized based on pH, temperature, agitation, time and initial glucose concentration. The morphological, physiological and molecular characterization was investigated for identification of the isolates. Among all the 10 ethanol producing isolates, the two highest producing isolates were selected for further studies. Both of them were motile and catalase positive but failed to hydrolyze gelatin and produce H_2S. Among them, isolate ZYM6 exhibited highest ethanol yield 6.28 gL^{-1} with optimum pH 6 and growth temperature of 30ºC. In addition, isolates ZYM6 and ZYM10 exhibited highest ethanol yield: 15.00 gL^{-1} and 12.00 gL^{-1} with xylose and tryptophan, respectively. Thus, the optimum condition for ethanol production was a medium characterized by pH 6, growth temperature of 30-35ºC for 24-48 hours and xylose and tryptophan as carbon and nitrogen sources. The results of morphological and physiological characteristics showed that isolates ZYM6 and ZYM10 belong to *Zymomonas*. Moreover, phylogenetic analyses based on *16S rRNA* sequences showed that isolates ZYM6 and ZYM10 were similar to *Zymomonas mobilis* with 99% homology. These native *Zymomonas* spp. can produce ethanol with high yield. In addition, xylose is a feasible feedstock for ethanol fermentation with high efficiency while using these isolates.

Keywords: Bacteria; Biotechnology; Ethanol production; Phylogeny; *Zymomonas*

Introduction

The increase in the prices of fuel and possibility of shortfalls has led to an extensive evaluation of alternative sources of energy to meet the global energy demand (1-3). Among liquid fuels that are

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currently considered, ethanol is particularly appealing since it is a renewable source when obtained from biomass, is easy to handle and distribute and is readily available (4). Microbial processes have been proved useful for production of alternate energy products from renewable resources (5).

Alcoholic fermentation is one of the most important examples. Ethanol is the most promising liquid fuel since it can be readily produced from various agriculture-based renewable materials (6). Currently, *Saccharomyces cerevisiae* is used as the major ethanol producing microorganism worldwide (7). Despite its expensive use, it has a number of disadvantages, such as high aeration cost, high biomass production and low temperature and ethanol tolerances (2, 8, 9). Therefore, efforts have been made to improve the existing technologies through the raw materials and alternate strains for ethanol production (10). *Zymomonas mobilis* has emerged as a potential bacterium for ethanol production. The studies have clearly demonstrated that it has a high specific rate of sugar uptake (11, 12), high ethanol yield (13), low biomass production and non-requirement to controlled addition of oxygen to maintain the viability of the cells (14). It is widely distributed in natural habitats and classified into the family Sphingomonadaceae. Members of this family are useful in industrial ethanol production (10). Since province of Mazandaran is located in a dump area, it is likely to have high diversity of ethanol producing bacteria. This study aims to isolate and identify ethanol producing bacteria from natural environments, optimize the ethanol production and evaluate their productivity.

Materials and methods

Screening and identification of bacteria

To isolate ethanol producing bacteria, various samples were collected from natural resources including fruits (apple, fig, grape, apricot, nectarine, orange, tangerine, pomegranate, peach, pear and cane) and plants (agave and sugarcane). Samples were crushed aseptically then inoculated into *Zymomonas* Sucrose Medium (ZSM) contained (gL^{-1}): sucrose, 20 g; yeast extract, 10 g; ammonium sulfate, 2 g; KH_2PO_4, 2 g; $MgSO_4$ $7H_2O$, 0.5 g; pH 6.8). The Durham tubes were incubated at 35ºC for 1-7 days (15). Those cultures produced CO_2 gas, plated out on RM medium (contain gL^{-1}: 20 g glucose, 10 g yeast extract, 2 g ammonium sulfate, 2 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 15 g agar, pH 6.8) supplemented with 0.083 mg mL^{-1} of Nistatin to inhibit the growth of fungi. Plates were incubated at 35ºC for 2 days under aerobic conditions. Colonies growing on RM medium were isolated for further studies (16).

For identification of the bacterial isolates, morphological and physiological characteristics were examined using the methods described in Bergey's manual of systematic bacteriology (17).

Ethanol production and assay

The isolates were evaluated for producing of ethanol in ZSM medium at 35ºC. After 48 hours incubation, the ethanol was assayed using both GC-Mass and dichromate colorimetric methods (18). The ethanol concentration that produced in ZSM culture was estimated using microprocessor based gas chromatograph equipped with flame ionization detector and DB-5 column. The injector, detector and oven temperature of gas chromatograph were maintained at 200, 210 and 100°C, respectively (2). In dichromate colorimetric method, the reaction mixture containing 1 mL sample, potassium dichromate 50 gL^{-1} and saturated diphenylcarbazide was heated at 90ºC for 5-15 mins until it turned brown. Then, 1 mL of sodium potassium tartrate (40%) was added for stabilization of the produced color. The absorbance was measured at 575 nm (19).

The ethanol yield (*Y*ps) was calculated as the actual ethanol produced and expressed as g ethanol per g sugar utilized (gg^{-1}). The volumetric ethanol productivity (*Q*p) and the percentage of conversion efficiency or yield efficiency (*E*y) were calculated by the following equations:

$$Qp=P/t$$

$$Ey=(Yps\times 100)/0.51$$

Where *P* is the actual ethanol concentration produced (gL^{-1}), t is the fermentation time (h) giving the highest ethanol concentration and 0.51 is the maximum theoretical ethanol yield of sugar consumption (20).

Optimization of culture medium and conditions

To find a suitable medium and condition for ethanol production, different carbon and nitrogen sources were examined (21). Different carbon sources including glucose, xylose, fructose, maltose, sucrose, ribose, galactose, mannose and arabinose were used at 20 gL^{-1} in RM basal medium. In addition, the effect of glucose on ethanol production was studied using different concentrations as 0.5, 1.0, 1.5 and 2.0 % (w/v).

Nitrogen sources for optimization process were yeast extract, peptone, cysteine, ammonium sulfate, alanine, arginine and tryptophan. The nitrogen sources were added at 10 gL^{-1} in RM basal medium.

In addition, the effects of temperature, initial pH, time of fermentation and agitation on ethanol production was examined. Isolates were cultivated at a range of temperatures 25, 30, 35, 40ºC; various pH 2, 4, 6 and 8; different fermentation time 24, 48, 72, 96 hours and various agitation rate 50, 100, 150, 200 rpm.

DNA extraction, PCR amplification and sequencing of the 16S rRNA gene

DNA was extracted from RM cultures using a standard bead beating method (22). DNA pellets were washed in absolute ethanol, air-dried and re-suspended in 30 μL^{-1} of ultrapure water (Sigma). PCR amplification of the *16S rRNA* gene was performed using the universal bacterial *16S rRNA* primers (PA 5′-AGA GTT TGA TCC TGG CTC AG-3′ and PH 5′-AAG GAG GTG ATC CAG CCG CA-3′) (23). PCR amplification was performed in a MJ Mini thermal cycler (Bio-Rad) and cycling conditions described by Edwards et al. (21). The thermocycler was programmed to denature at 95ºC for 5 mins followed by 35 cycles of 1 mins at 95ºC (denaturation), 1 min at 50ºC (annealing), and 72ºC for 2 mins (extension), with a final extension step for 5 mins at 72ºC. PCR reaction mixtures (50 µL) contained 50 ng of DNA template, 2 µL of each primer (10 pmol μL^{-1}), 25 µL of PCR Master Mix (Promega) and 19 µL ultrapure water (Sigma). PCR products were analyzed by electrophoresis on 0.8% (w/v) agarose gels in 1X TAE buffer [20 mM Tris, 10 mM sodium acetate, 0.5 mM EDTA, pH 7.4] at 100 V for 30 mins using a Bio-Rad DNA sub cell (Bio-Rad) (24). The PCR products were purified using a GeneJet PCR purification kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. Purified products were sequenced by GATC Biotech (Germany). The *16S rRNA* gene sequences were analyzed against those available from the National Centre for Biotechnology Information (NCBI) database using the BLAST search system to identify the most similar sequences.

Phylogenetic analysis of 16S rRNA gene sequences

The *16S rRNA* sequences were analysed for anomalies using the Chromas Lite (2.01) software package, then assembled using the CAP contig program in Bioedit (7.1.3.0). FASTA and BLAST subroutines were used to determine the closest relatives in the GenBank database (25). The sequences were compared with other closely related bacterial sequences from GenBank using the FASTA algorithm (26). Sequences were aligned and analysed with ClustalX program (26, 27). The phylogenetic package MEGA6 was used to carry out the nucleotide substitution model of Jukes and Cantor in order to obtain distance matrices (28). Phylogenetic trees were then constructed using the neighbour-joining method (29).

16S rRNA gene sequence accession numbers

The 16S rRNA gene sequences of the *Zymomonas* spp. isolates described in this study have been deposited under following accession numbers: isolate ZYM6, KF836761 and isolate ZYM10, KF836762.

Results

A total of 10 isolates were selected as ethanol producing bacteria. They were Gram-negative and Gram-positive, circular or rod-shaped, entire-edged and cream-color with non-pigmented colonies (Fig. 1). The isolates were tested for ethanol production in the RM medium. These isolates were inoculated on RM broth and a total of 10 isolates showed signs of gas production. These bacteria isolated from pomegranate, apple, grape, peach, pear, sap of plants

showed a highest productivity of >10% ethanol. Among all 10 isolates, two that produce high gas production in Durham tube were selected for further studies.

Effect of pH

To check the effect of pH on ability of isolates ZYM6 and ZYM10 to produce ethanol, pH of the RM medium was adjusted from 2 to 8 then incubated at 35ºC in static conditions for 48 hours. The results indicated that maximum ethanol was produced at pH 6–8 (Table 1). ZYM6 and ZYM10 exhibited highest ethanol yield 6.28 gL^{-1} and 7.49 gL^{-1} at pH 6. In addition, the results obtained from Table 1 revealed that ZYM6 and ZYM10 were unable to produce ethanol at pH 8. However, ethanol production of the isolate ZYM6 was 3.00 g L^{-1} at pH 2. The isolates ZYM6 and ZYM10 produced 4.30 g L-1 and 5.65 gL^{-1} ethanol at pH 4, respectively.

Effect of initial glucose concentration

To find out optimum sugar level for fermentation, batch fermentation was carried out with varying levels of glucose. Ethanol production by two isolates in different glucose concentrations is summarized in Table 1. The maximum efficiency of fermentation was observed at 1.5% glucose by ZYM6 and ZYM10 with 8.56 gL^{-1} and 5.00 gL^{-1} ethanol, respectively. In addition, the isolates ZYM6 and ZYM10 produced 7.40 gL^{-1} ethanol at 2.0% glucose, respectively. The results indicated that all isolates were able to produce ethanol at low initial glucose concentration 0.5%.

Effect of growth temperature

To determine the effect of temperature on ethanol production, the isolates were cultured at different temperature. The results obtained from Table 1 demonstrated that the optimum growth temperature was 30ºC, and high ethanol (6.80 gL^{-1}) was produced by ZYM6 at 30ºC. In addition, the isolate ZYM10 produced 5.75 gL^{-1} ethanol at the same temperature. All isolates grown at 25 and 40ºC showed no ethanol production.

Effect of fermentation time

To study the effect of time on ethanol production, RM broth was inoculated with activate culture of ZYM6 and ZYM10 then incubated static condition at 35ºC for 24, 48, 72 and 96 hrs. The results revealed that the ethanol production was increased over time (Table 1). Ethanol production by ZYM10 was raised due to increasing fermentation time from 24 to 72 hrs, whereas it was decreased after 96 hrs incubation. More ethanol was produced by ZYM10 after 24 hours and ZYM6 after 48 hours incubation with 5.70 gL^{-1} and 5.74 gL^{-1} ethanol, respectively.

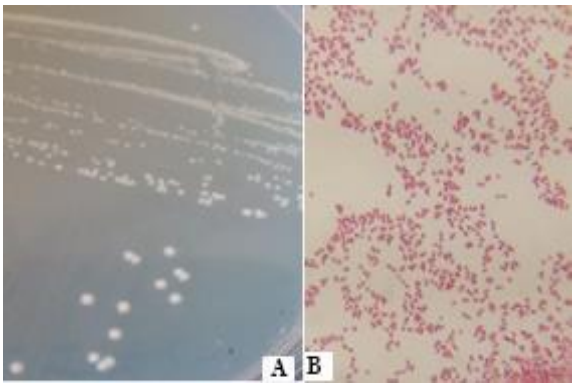

Figure 1. Colonies of pure isolate ZYM6 on RMA after 24 hours (A), and rod shaped, Gram negative ZYM6 (B).

Effect of agitation

Results from Table 1 indicating agitation played an important role in producing of ethanol. Ethanol production and biomass concentration was strongly improved by increasing agitation. The biomass was increased with raising agitation speed from 50 to 200 rpm. These results were correlated with ethanol production rate when agitation increased from 50-150 rpm. Maximum biomass concentration was achieved after 48-72 hrs incubation at 50-100 rpm agitation, while maximum biomass was observed after 24-48 hrs when agitated was at 150 and 200 rpm. The maximum ethanol level of the culture ZYM6 was 5.49 gL^{-1}, agitated at 150 rpm. Both ZYM6 and ZYM10 have been able to produce ethanol in all level of agitation. There was no doubt that agitation would strongly improve ethanol concentration from the results gained from Table 1.

Table 1. Effect of pH, fermentation time, initial glucose concentration, temperature and agitation on ethanol production

Isolates	Effect of time		Effect of pH		Effect of temperature		Effect of initial glucose concentration		Effect of agitation	
	Time (h)	Ethanol (gL^{-1})	pH	Ethanol (gL^{-1})	Temperature (˚C)	Ethanol (gL^{-1})	Concentration (%)	Ethanol (gL^{-1})	RPM	Ethanol (gL^{-1})
ZYM6	24	0	2	3.00±0.13	25	1.62±0.04	0.5	1.21±0.01	50	1.00±0.01
	48	5.74±0.1	4	4.30±0.13	30	6.80±0.08	1.0	4.74±0.02	100	1.17±0.1
	72	4.74±0.15	6	6.28±0.12	35	6.28±0.05	1.5	8.56±0.1	150	5.49±0.01
	96	1.00±0.05	8	0	40	0	2.0	7.40±0.05	200	3.95±0.03
ZYM10	24	5.70±0.1	2	0	25	0	0.5	1.00±0.01	50	1.60±0.1
	48	4.65±0.1	4	5.65±0.05	30	5.75±0.02	1.0	1.27±0.02	100	3.00±0.15
	72	4.50±0.05	6	7.49±0.25	35	4.34±0.04	1.5	5.00±0.2	150	4.00±0.05
	96	0	8	0	40	0	2.0	5.30±0.1	200	0

Effect of agitation

Results from Table 1 indicating agitation played an important role in producing of ethanol. Ethanol production and biomass concentration was strongly improved by increasing agitation. The biomass was increased with raising agitation speed from 50 to 200 rpm. These results were correlated with ethanol production rate when agitation increased from 50-150 rpm. Maximum biomass concentration was achieved after 48-72 hrs incubation at 50-100 rpm agitation, while maximum biomass was observed after 24-48 hrs when agitated was at 150 and 200 rpm. The maximum ethanol level of the culture ZYM6 was 5.49 gL^{-1}, agitated at 150 rpm. Both ZYM6 and ZYM10 have been able to produce ethanol in all level of agitation. There was no doubt that agitation would strongly improve ethanol concentration from the results gained from Table 1.

Effect of carbon and nitrogen sources

To study the effect of carbon and nitrogen sources on ethanol producing ability of bacterial isolates, RM broth was supplemented with different carbon and nitrogen sources then incubated static condition at 37ºC for 48 hours. The results in Table 2 showed that the best carbon source for most isolates was xylose. The isolates, ZYM6 and ZYM10 exhibited highest ethanol yield 15 gL^{-1} and 12 gL^{-1} with xylose, respectively. In addition, the results obtained from Table 2 revealed that most isolates were able to produce high ethanol when consumed five different carbon sources.

The results of study on nitrogen sources obtained from Table 2 demonstrated that the highest amount of ethanol was produced by ZYM6 and ZYM10 (4.87 gL^{-1}) with tryptophan. The ethanol yield (*Y*ps) was calculated as the actual ethanol produced. The volumetric ethanol productivity (*Q*p) and the percentage of conversion efficiency or yield efficiency (*E*y) were calculated (Table 3).

Identification and morphological analysis

Morphological and physiological characteristics of two isolates are summarized in Table 4. Both isolates were occurred as single or in pairs and were motile. The same characteristics were observed in the reference organism, *Z. mobilis* PTCC1718. When plates were incubated aerobically, the resulting colonies were smaller than those incubated anaerobically. The average colony diameter for isolates grown aerobically, after 48 hrs incubation, was 1.0-1.2 mm while those isolates incubated anaerobically ranged from 1.8 to 2.0 mm. Physiological and biochemical tests showed uniform reaction of all isolates (Table 4). Two isolates were catalase positive, failed to hydrolyze gelatin, and did not produce H_2S. The results of morphological and physiological characteristics showed that ZYM6 and ZYM10 belong to the genus *Zymomonas.* Moreover, *16S rRNA* sequencing and phylogenetic analyses revealed that ZYM6 and ZYM10 were similar to *Zymomonas mobilis* with 99% homology (Fig. 2).

Table 2. Effect of different carbon and nitrogen sources on ethanol producing isolates

Isolate	Effect of carbon source		Isolate	Effect of nitrogen source	
	Carbon Source	Ethanol (gL^{-1})		Nitrogen Source	Ethanol (gL^{-1})
	Sucrose	4.00±0.1		Cysteine	0
	Glucose	3.50±0.12		Alanine	0
	Fructose	0		Arginine	0
ZYM6	Arabinose	0	**ZYM6**	Tryptophan	3.40±0.01
	Xylose	15.00±0.06		Ammonium sulfate	0
	Mannose	0		Peptone	2.10±0.01
	Ribose	1.34±0.02		Yeast extract	0
	Sucrose	0		Cysteine	0
	Glucose	6.00±0.1		Alanine	0
	Fructose	0		Arginine	0
ZYM10	Arabinose	3.00±0.11	**ZYM10**	Tryptophan	3.40±0.01
	Xylose	12.00±0.1		Ammonium sulfate	0
	Mannose	2.00±0.1		Peptone	3.00±0.11
	Ribose	2.10±0.1		Yeast extract	0

Table 3. Maximum ethanol and theoretical yield of ethanol producing isolates

Strain	Maximum ethanol (gL^{-1})	*Y*ps	Theoretical yield	Time of fermentation (h)
ZYM6	15.00	0.75	100%	24
ZYM10	12.00	0.60	100%	48

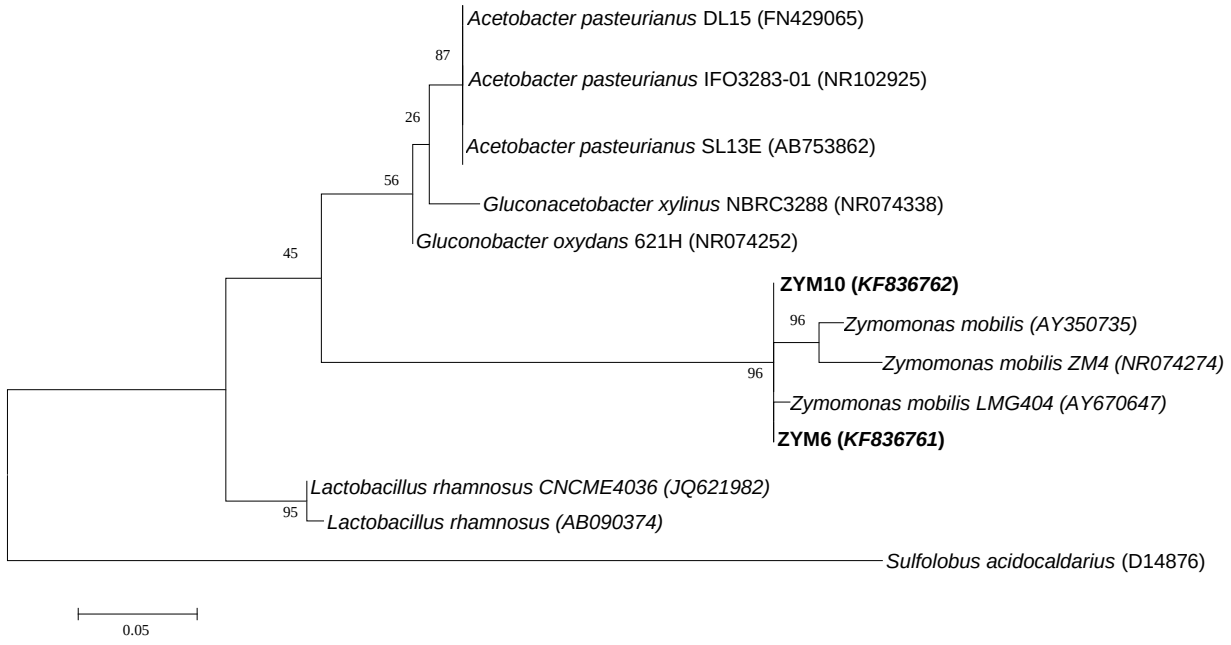

Figure 2. Neighbour-joining tree showing phylogenetic relationships of *16S rDNA* sequences of isolates ZYM6 and ZYM10 to closely related sequences from GenBank. Bootstrap values of >50% are shown. Scale bars indicate Jukes-Cantor distances.

Discussion

For industrial ethanol production, several properties of the fermenting organism are important for minimizing the costs involved. An ethanologenic microorganism capable of fermenting the sugars from natural biomass through a saccharification process is essential for secondary bioethanol production. The eventuality to isolate different species from the samples increases irrespective to their relative presence. In this study, the number of strains isolated able to grow in low-cost-row and increased level of ethanol production was high. Morphological examination of the isolates revealed Gram-negative, plump rod cells with distinct rounded ends. No endospores were observed. The same characteristics were observed in the reference organism, *Z. mobilis* ATCC10988. Such characteristics were also reported in an earlier study (15). In the present study, the isolated bacteria were able to grow at 15% glucose concentration of RM medium. Production of >10% ethanol at the initial stage of isolation was promising. Ethanol producing bacteria are characterized by the ability to oxidize sugars incompletely, and a common feature to most of them is the ability to produce ethanol.

Temperature optimization is a major factor for any biotechnological process because of the over temperature effect on bacterial deactivation and growth. This deactivation is attributed to the essential enzyme denaturation, membrane damage that causes cellular constituent scattering and making the organism more sensitive to the toxic effect of acetic acid (20). Thus, *Z. mobilis* showed maximum ethanol production and sugar utilization at 30ºC. Further increase in temperature had shown an inhibitory effect on the ethanol production as well as sugar utilization abilities of the test isolate (20). It was also observed that the decrease in ethanol production was less and between 30-35ºC, in contrast to sharp decrease between 35-40ºC (30). The decrease in membrane phospholipid content may be responsible for the unique thermal sensitivity of cells grown at high temperature 41ºC (31). The decrease in the cell viability and final ethanol concentration with the increased temperature from 30 to 40ºC in batch culture has also been found in *Z. mobilis* ATCC10988 (32). In another study, *Z. mobilis* CP4 has shown optimal ethanol production from sugarcane molasses at 34ºC (33). In our study, the optimum growth temperature was found to be 35ºC. It is clear from the observations recorded during the course of the study that the isolated bacteria had optimal production of ethanol at 30-35ºC. Therefore, with increasing temperature, the ethanol production was decreased.

The results of this research demonstrated that most isolates were able to produce high ethanol when consuming different carbon sources. However, it performed better on xylose as compared to glucose in terms of ethanol production, sugar utilization as well as ethanol and temperature tolerance. Xylose is a second most abundant sugar component of lignocellulose besides glucose. Efficient fermentation of xylose is important for the economics of biomass-based bio refineries. Cheap materials, low-cost processing and high ethanol productivity are the main considerations for most ethanol fermentation (34, 35). Xylose can be used as cheap and abundant raw material for fermentation to ethanol with high ethanol productivity (36). Overall, this work demonstrated that xylose was an excellent carbon source for ethanol fermentation. The isolates grow on D-xylose as the sole carbon/energy sources and ferment these pentose sugars to ethanol in high yield. In this study, the fermentation efficiency with high concentration of sugar (15-20 gL^{-1}) was achieved over 90%.

Lower pH in the media is regarded to minimize the occurrences of contamination. Rogers et al. (37) showed that the growth optimal pH for ethanol producing bacteria was 6. Findings from present study, indicated an optimum pH for both growth and ethanol production to be at a wider range (between 6 and 8). This result is in agreement with previous studies (37). However, the tolerance to low pH is strongly dependent on other parameters such as ethanol concentration and oxygen availability.

The results of this research demonstrated that most isolates were able to produce high ethanol when consumed agitation. The agitation was demonstrated here to be adequate to support the growth of isolated ethanol producing bacteria and ethanol production. Aeration can be useful for the development and function of microorganisms by improving the mass

transfer characteristics with respect to substrates (38). Aeration helps to maintain the concentration gradient between the inside and outside of cells in the fermentation broth. Concentration gradient works in both directions; through better diffusion helping to maintain a satisfactory supply of sugars and other nutrients to the cells, which facilitates the removal of gases and other byproducts of catabolism from the microenvironment of the cells.

These native isolated *Zymomonas* spp. can produce ethanol with high yield. Owing to its low cost and no inhibition to ethanol production, the xylose is a feasible feedstock for ethanol fermentation with high efficiency using these isolates. Therefore, these organisms are projected as potential ethanol producer candidate for further commercial exploitation in industry to produce bioethanol and biofuel.

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